Exhibit E

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Identification of Two Forms (31–33 and 48 kD) of the Urinary Soluble p55 Tumor Necrosis Factor Receptor That Are Differentially N- and O-Glycosylated

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ABSTRACT

The structure and the activity of urinary soluble TNF receptor type 1 (sTNF-R1), isolated from the urine of normal individuals, has been characterized and compared with that of recombinant sTNF-R1 expressed in CHO cells and with that of a nonglycosylated form expressed in *Escherichia coli*. Urinary sTNF-R1 was resolved in a major band of 31–33 kD and in a 48 kD band (less than 5% of total) by reducing SDS-PAGE; CHO sTNF-R1 was resolved in two bands of 29 and 31 kD. All bands were recognized by various anti-sTNF-R1 antibodies as well as by TNF-α in western and ligand blotting assays. No cross-reaction was observed with anti-TNF-R2 antibodies. N- and O-glycosylation studies indicated that (1) the 29–31 kD recombinant form as well as the 31–33 kD urinary form are N-glycosylated; (2) the differences between the 29–31 and 31–33 kD recombinant and natural products are mainly related to differences in the N-linked sugar content; and (3) the 48 kD sTNF-R1 isolated from urine also contains O-linked sugars. The urinary sTNF-R1 antigen mixture was able to inhibit TNF-α cytotoxicity with a potency comparable to that of nonglycosylated *E. coli* sTNF-R1. At variance, urinary sTNF-R1 was able to inhibit TNF-β sevenfold more efficiently than *E. coli* sTNF-R1. In conclusion, two subtypes of sTNF-R1 have been isolated from urine: a main N-glycosylated form of 31–33 kD and a N- and O-glycosylated form of 48 kD that appears to be a minor constituent of the urinary sTNF-R1 antigen.

INTRODUCTION

TUMOR NECROSIS FACTOR α (TNF-α) is a macrophage-derived cytokine able to exert a wide variety of different *in vitro* and *in vivo* activities. Originally identified for its cytotoxic effects on tumor cells, TNF-α was found later to be involved in the regulation of inflammatory and cellular immune responses, as well as in the pathogenesis of various human diseases, including endotoxic shock, cachexia, rheumatoid arthritis, and inflammatory tissue distruction (see Refs. 1–3 for reviews). The molecular basis of the pleiotropic activity of TNF-α has been a matter of intensive investigation in the past few years. Analysis of the structure of TNF-α in the crystalline form and in solution have shown that TNF-α is a tightly packed homotrimeric protein[4–9] that slowly dissociates at picomolar concentrations.[10–12] Several lines of evidence suggest that trimeric TNF-α is the bioactive form and that TNF-α polyvalence is necessary for triggering cellular cytotoxicity.[9–11,13]

Two membrane receptors, designated TNF-R1 and TNF-R2, have been identified and are thought to mediate and regulate most TNF-α activities.[14] These receptors are able to bind with high affinity TNF-α as well as TNF-β, a TNF-α-related cytokine. Protein structure and cDNA analysis revealed that TNF-R1 and TNF-R2 are characterized by different antigenic properties, molecular mass (55–60 and 75–80 kD, respectively), and glycosylation patterns.[15–21] The extracellular domains, 182 residues long in TNF-R1 and 235 residues in TNF-R2, contain four cysteine-rich domains sharing significant sequence homology.[15,16] In contrast, no homology occurs at the cytoplasmic domains, suggesting that the two receptors activate different intracellular signal pathways. Cross-linking of membrane receptors with polyvalent antibodies specific for

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TNF-R1 or TNF-R2 was sufficient to trigger many TNF-α activities, suggesting that clustering of each receptor may provide sufficient signal for triggering TNF-α effects. For instance, antibodies against TNF-R1 were able to trigger cytotoxicity, fibroblast proliferation, and synthesis of prostaglandin E_2 in a TNF-α-like manner.[22-24]

Two soluble(s) TNF binding proteins corresponding to extracellular domain fragments of the membrane receptors have been isolated from the urine of normal subjects and of patients with fever or with renal failure,[25-29] as well as from the blood of cancer patients.[30] Increased levels of sTNF-R1 and sTNF-R2 have been detected in the urine of febrile patients and in the sera of patients with renal failure, burns,[31] solid tumors,[32] rheumatoid arthritis,[33] and meningococcaemia,[34] as well as in uremic and long-term hemodialysis patients.[35] These proteins are thought to regulate the bioavailability of active TNF-α through inhibition of binding to membrane receptors or, in the opposite way, by stabilizing the TNF-α quaternary structure of bioactive trimers.[36]

To understand better the function of membrane and soluble TNF receptors in mediating the pleiotropic TNF-α and TNF-β activities, further characterization of receptor structures is necessary. To this aim, we have characterized the structure of the urinary sTNF-R1 antigen and compared it with that of a soluble form of the TNF-R1 produced by recombinant DNA techniques. We show that at least two subtypes of sTNF-R1 can be isolated from the urine of normal individuals: a N-glycosylated 31–33 kD form, similar in size to the previously described sTNF-R1,[25-30] and a N- and O-glycosylated form of 48 kD, both functionally able to bind TNF-α.

MATERIALS AND METHODS

Materials and cell lines

Human recombinant TNF-α, expressed in yeast, was from Esquire Chemie AG (Zurich, Switzerland); rabbit anti-TNF-α antiserum and rat antihuman TNF-R2 (p75), neuraminidase (E.C. 3.2.1.18), and *O*-glycanase (E.C. 3.2.1.97) were from Genzyme Corporation (Boston, MA). Recombinant sTNF-R1 and sTNF-R2 (expressed in *Escherichia coli*) were from R&D Systems (UK). Rabbit antihuman sTNF-R1 was a generous gift from Dr. M. Lantz (University of Lund, Sweden). sTNF-R (60 kD) enzyme-linked immunosorbent assay (ELISA) kit was from Bender MedSystem (Vienna, Austria). The 96-well polyvinyl chloride microtiter plates (Falcon MicroTest III flexible assay plates) were obtained from Becton Dickinson and Co. (Oxnard, CA). Bovine serum albumin (BSA, Cohn fraction V), normal goat serum (NGS), polyoxyethylene sorbitan monolaureate (Tween 20), goat antirabbit IgG peroxidase (GAR-HRP), goat antimouse IgG (GAM), goat antimouse IgG-HRP (GAM-HRP), and goat antimouse IgG biotin (GAM-B) were from Sigma Chemical Co. (St. Louis, MO). Streptavidin–alkaline phosphatase conjugate (STV-AP) was from Calbiochem (San Diego, CA). Streptavidin-peroxidase (STV-HRP) was from Janssen Biochimica (Beerse, Belgium). DIG glycan detection kit, 2,2'-azinodi-(3-ethylbenzthiazoline sulfonate) (ABTS) chromogenic solution, and peptide-*N*-glycosidase F (E.C. 3.2.2.18) were from Boehringer Mannheim Italia SpA (Milan, Italy). 3(4,5-Dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide was from Calbiochem (San Diego, CA). Cell culture reagents, media, and fetal calf serum (FCS) were from GIBCO (Gaithersburg, MD). Murine L-M cells (American Type Culture Collection CL12) were cultured in Eagle's minimum essential medium, 5% FCS, and 2 mM glutamine and maintained at 37°C and 5% CO_2. All other reagents were analytic-grade products from Carlo Erba (Milan, Italy).

Preparation of anti-sTNF-R1 monoclonal antibodies (MAbs)

MAbs 7H3 (IgG_1), 4E10 (IgG_1), and 9B11 (IgG_{2a}), specific for sTNF-R1, were prepared as described[37] and purified from cell culture media by precipitation with ammonium sulfate (50% saturation), followed by affinity chromatography on a protein A-agarose column (2.5 × 3 cm), equilibrated with 3 M sodium chloride and 1.5 M glycine, pH 8.9, and eluted with 0.1 M sodium citrate, pH 5.0. The final products were >90% pure as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

sTNF-R1 ELISA

Detection of sTNF-R1 in urine, urinary extracts, and cell culture medium was carried out as follows: polyvinyl chloride microtiter plates were coated with MAb 7H3 by incubating overnight at 4°C with 10 μg/ml of 7H3 solution in 0.15 M sodium chloride and 0.05 sodium phosphate buffer, pH 7.3 (PBS; 100 μl/well). All subsequent steps were carried out at room temperature. After washing three times with PBS containing 0.05% vol/vol Tween 20 (PBS-T), the plates were blocked by incubating with PBS containing 0.5% BSA and 0.05% Tween 20 (blocking buffer) (200 μl/well, 2 h) and washed again with PBS-T. Then, sTNF-R1 standard solutions (made up with sTNF-R1 purified from urine according to method A and quantified using the sTNF-R [60 kD] ELISA kit from Bender MedSystem) or samples diluted with PBS containing 50% FCS were mixed 2:1 with biotinylated MAb 9B11 (1:400 in blocking buffer, final concentration 330 ng/ml) and preincubated for 1 h. Then, 150 μl of each mixture was added to different wells and further incubated 2 h under gentle mixing with a plate vortex. The plates were washed eight times by emptying and filling with PBS-T and further incubated for 1 h with STV-HRP solution (1:10,000 in blocking buffer, 100 μl/well). After a final wash with PBS-T, each well was incubated for 30 minutes with 200 μl ABTS chromogenic solution and the absorbances at 405 nm were measured.

Cloning and expression of recombinant sTNF-R1 in CHO cells

The cDNA corresponding to the extracellular domain of TNF-R1 was amplified by polymerase chain reaction from U937 cell total mRNA as a 603 base pair fragment, starting from the first ATG codon, as described.[38] The cDNA was cloned into the expression vector pCDNAINeo (Invitrogen) and transfected in CHO cells. Among several clones selected in Ham's F12, 5% FCS, 1% glutamine, 100 IU penicillin, 100 μg/ml of streptomycin, and 0.6 mg/ml of G418, one clone was

chosen for its productivity and maintained in the absence of G418 without loss of productivity. Conditioned medium, collected from transformed cells at 4–5 day intervals, contained 20–30 ng/ml of sTNF-R1 by sTNF-R1 ELISA.

Purification of urinary and recombinant (CHO) sTNF-R1

sTNF-R1 was purified from urine using two methods, the first based on affinity chromatography on TNF-α-agarose (method A) and the second based on affinity chromatography on a MAb 7H3-agarose column (method B). TNF-α- and 7H3-agarose columns (1 × 3 cm) were prepared by coupling TNF-α (2 mg) and 7H3 (10 mg) to activated CH-Sepharose (Pharmacia), according to the manufacturer's instructions (coupling efficiency > 90%).

Method A: A crude extract of urinary proteins (1.6 liters; kindly supplied by Allevamento S. Fiorano, Burago, Italy), corresponding to about 160 liters urine, was dialyzed overnight against PBS and concentrated to 520 ml by ultrafiltration through a polysulfone membrane (PTGC-10,000 NMWL, Millipore) using a Minitan apparatus (Millipore). The product was loaded onto the TNF-α-agarose column (flow rate, 40 ml/h) and washed with PBS until the absorbance of the effluent reached the baseline. The column was then eluted with 0.2 M glycine buffer, pH 3.5. Peak fractions were collected, pooled, and further purified by reversed-phase high-performance liquid chromatography (HPLC) on a Pro-RPC HR5/10 column (Pharmacia) using the following conditions: buffer A, 0.1% trifluoracetic acid in water, buffer B, 0.1% trifluoracetic acid in acetonitrile, 0% B for 20 minutes; linear gradient 0–70% B in 50 minutes; 100% B for 20 minutes; flow rate, 0.3 ml/h. Peak fractions were pooled and vacuum dried. The product was dissolved with 100 μl distilled water and kept at −20°C as a stock solution for further studies. The content of sTNF-R1 antigen in the final product was quantified using a commercially available ELISA kit (Bender).

Method B: sTNF-R1 was purified from urinary extracts or from recombinant CHO cell supernatants essentially using the same conditions described for method A except that a 7H3-agarose column, instead of the TNF-α-agarose column, was used. In this case sTNF-R1 was desorbed from the column using 0.2 M glycine, pH 2.8.

SDS-PAGE and western and ligand blot analysis

SDS-PAGE was carried out under reducing and nonreducing conditions on Phast-Gels gradient 8-25 or 10-15 gels using a Phast-System (Pharmacia) according to the manufacturer's instructions.

Electrophoretic transfer of proteins to 0.45 μm nitrocellulose membranes (BioRad) was carried out using the Phast-Transfer semidry transfer kit (Pharmacia) in 15 minutes at 15°C, using 25 mM Tris and 192 mM glycine, pH 8.3, containing 20% methanol as transfer buffer and 25 mA/gel, according to the manufacturer's instructions.

Western blot analysis with mouse monoclonal antibodies was carried out as follows: membranes were washed for 5 minutes with 100 mM Tris-HCl and 150 mM sodium chloride, pH 7.5 (TBS), and blocked with freshly prepared TBS containing 0.5% blocking reagent (Boehringer, TBS-BR; 1 h at room temperature). After a rapid wash (1 minute) with TBS, membranes were immersed in 2 μg/ml of anti-TNF-R1 antibody solutions in TBS-BR containing 3% NGS and 0.1% BSA (TBS-BRNB) and left to incubate under gentle mixing for 1 h. Membranes were then washed three times (10 minutes each) with TBS-BRNB, further incubated for 1 h with a GAM-B solution (1:400 in TBS-BRNB), and washed again. The membranes were then immersed in a STV-AP solution (1:500) in TBS-BRNB for 1 h and washed three times as before and once (10 minutes) with 100 mM Tris-HCl, 100 mM sodium chloride; and 50 mM magnesium chloride, pH 9.5. Bound alkaline phosphatase was detected by immersing the membrane in a bromochloroindolyl phosphate and nitroblue tetrazolium chromogenic solution prepared as described.[39]

Western blot analysis with anti-sTNF-R1 rabbit polyclonal antiserum was carried out in a similar way using GAR-HRP as detecting reagent.

Ligand blot analysis of blotted membranes was carried out essentially as described for the western blot analysis except that TNF-α (2 μg/ml, 1 h), rabbit anti-TNF-α antiserum (1:500, 30 minutes), GAR-B (1:500, 30 minutes), and STV-AP (1:500, 30 minutes) in TBS-BRNB were used as detecting reagents.

Detection of carbohydrates on blotted membranes were carried out using the DIG glycan detection kit according to the manufacturer's instruction. This method is based on reaction of oxidized sugars with digoxigenin-hydrazide followed by detection, on nitrocellulose filters, with alkaline phosphatase–anti-digoxigenin antibody conjugate.

RESULTS

Preparation of urinary and recombinant (CHO) sTNF-R1

Natural sTNF-R1 was purified from urinary extracts using two different methods relying on affinity chromatography on TNF-α-agarose (method A) and on MAb 7H3-agarose (method B). The final products were subjected to reversed-phase HPLC and dried. As shown in Fig. 1, the materials eluted from the TNF-α- and 7H3-agarose columns were characterized by different compositions: in particular, three main peaks were observed with the product purified by TNF-α-agarose chromatography (peaks a), whereas a single peak was observed with the product purified by 7H3-agarose chromatography (peak b). Measurement of the TNF-α neutralizing activity of peak fractions, using the cytolytic assay on L-M cells, showed that the first and the second peaks of a as well as the single peak of b contained TNF-α inhibitors; the third peak was inactive. Moreover, only the first peak of both chromatograms was found to contain immunoreactive sTNF-R1 by ELISA, suggesting that the second peak is related to another TNF-α inhibitor, possibly the sTNF-R2.[27] Because traces of bioactive TNF-α were found in the product purified by affinity chromatography on TNF-α-agarose columns, sTNF-R1 prepared by method A was only used as antigen for the preparation of all monoclonal antibodies used throughout this work and for sTNF-R1 ELISA standard-

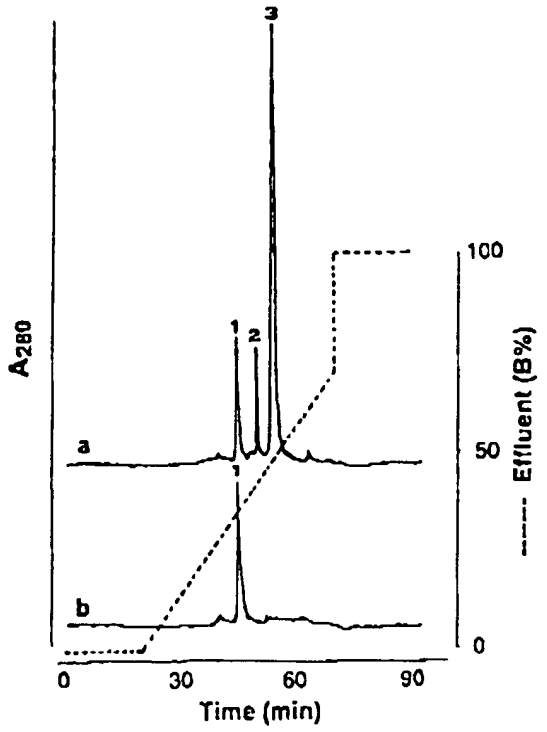

FIG 1. Reversed-phase HPLC of urinary sTNF-R1. Urinary sTNF-R1 purified by affinity chromatography on TNF-α-agarose (a) and on 7H3-agarose (b; see Results).

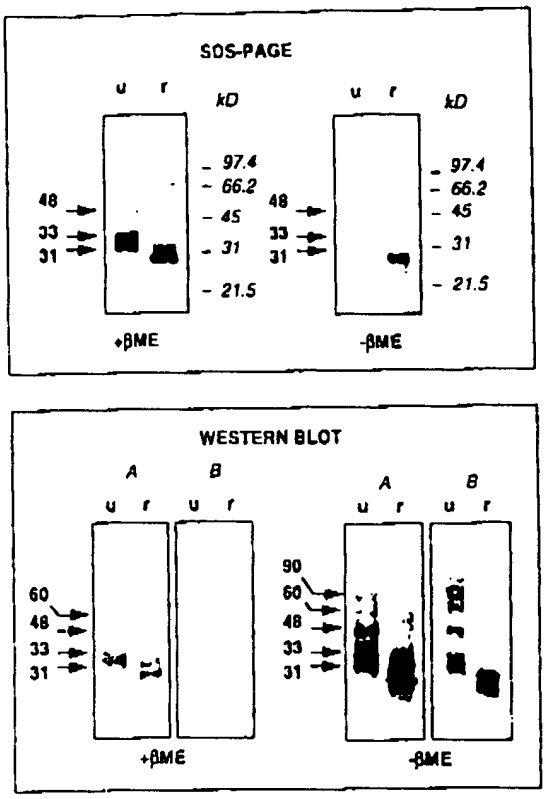

FIG. 2. SDS-PAGE and western blot analysis of urinary and recombinant sTNF-R1. Lanes u, urinary sTNF-R1; lanes r, recombinant sTNF-R1. SDS-PAGE was carried out under reducing (+βME, β-mercaptoethanol) and nonreducing (−βME) conditions. Western blot analysis (lower panels) was carried out with rabbit anti-sTNF-R1 polyclonal antibodies (A) and MAb 7H3 (B). Molecular markers were rabbit muscle phosphorylase b (97.4 kD), BSA (66.2 kD), hen egg white ovalbumin (45 kD), bovine carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD).

ization. All subsequent characterization studies were carried out with natural and recombinant sTNF-R1 purified by method B. Using this method, about 50 μg sTNF-R1 was recovered from 160 liters urine. A similar amount of recombinant sTNF-R1 was purified from 2.2 liters CHO cell supernatants transfected with the cDNA coding for the extracellular domain of TNF-R1.

Characterization of urinary and recombinant sTNF-R1 molecular weights

The molecular weights of urinary and CHO sTNF-R1 were characterized by SDS-PAGE and western blot analysis. To this purpose, monoclonal antibodies against distinct epitopes located within (MAb 7H3) and outside (MAb 4E10 and 9B11) the TNF-α binding site of sTNF-R1 and also able to recognize membrane-bound TNF-R1[37] and rabbit polyclonal antibodies were used.

Reducing SDS-PAGE of urinary sTNF-R1 (purified by method B) revealed a major diffused band of 31–33 kD and a minor 48 kD band (less than 5% of total; Fig. 2, upper panel, lane u). In contrast, only two 29–31 kD bands were observed with CHO sTNF-R1 (lane r). All these bands were immunoreactive with rabbit anti-sTNF-R1 antiserum by western blot analysis (lower panel, left). Similar bands, although more diffused, were observed under nonreducing conditions by SDS-PAGE as well as by western blotting with rabbit polyclonal antiserum or with MAb 7H3 (lower panel, right), MAb 4E10, and MAb 9B11 (not shown). In this case, however, additional bands of approximately 60, 90, and 120 kD were observed with urinary sTNF-R1. No bands were observed when anti-sTNF-R2 or irrelevant antibodies were used as negative controls or when the molecular marker mixture was blotted in place of sTNF-R1 (not shown). These results indicate that all bands observed are immunologically related to sTNF-R1.

Several bands > 60 kD corresponding to multiples of the 29–31 kD band were also observed in some cases by western and ligand blot analysis of urinary and CHO sTNF-R1, depending on the way the sample was prepared before analysis. For instance, the results of a ligand blot of boiled and nonboiled recombinant and urinary sTNF-R1 under nonreducing conditions using TNF-α as probe are reported in Fig. 3. As shown, a "ladder" of bands corresponding to multiples of the 29–31 kD ban can be observed after boiling samples (4–5 μl) for 3–5 minutes before analysis, but only the 29–31 kD doublet was observed when the sample was not boiled. Similar results were obtained with boiled and nonboiled urinary sTNF-R1, although in this case an additional 48 kD band was observed in both

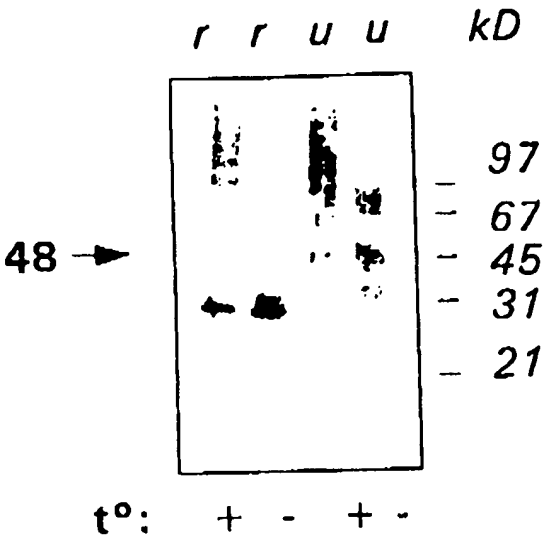

FIG. 3. Ligand blot of recombinant and urinary sTNF-R1 with TNFα. Lanes u, urinary sTNF-R1; lanes r, recombinant sTNF-R1. Samples (4 μl) in 100 mM Tris, 20 mM EDTA, and 5% SDS (wt/vol) were boiled (+) or not (−) and analyzed by SDS-PAGE under nonreducing conditions. Ligand blotting was carried out as described in Materials and Methods.

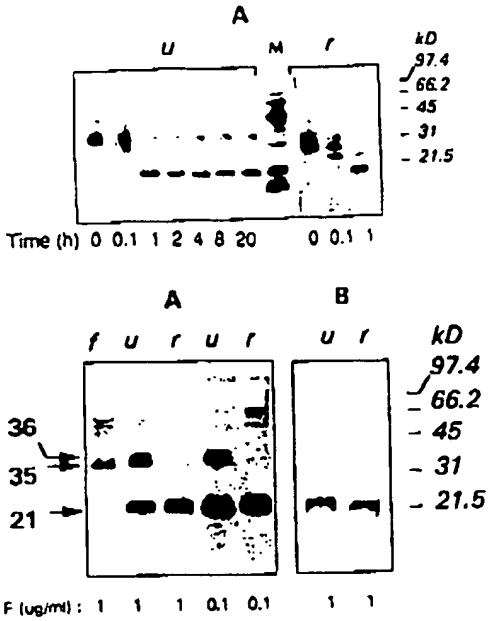

FIG. 4. Reducing SDS-PAGE and western blot analysis of urinary and recombinant sTNF-R1 after enzymatic treatment with peptide-*N*-glycosidase F. (A) SDS-PAGE; (B) western blot analysis with rabbit anti-TNF-R1 polyclonal antibodies; lanes u, urinary sTNF-R1; lanes r, recombinant sTNF-R1; lane f, peptide-*N*-glycosidase F alone, 1 μg/ml; lane m, molecular weight markers (see Fig. 2). Urinary or recombinant sTNF-R1, 100 μg/ml, in 0.5% wt/vol SDS, 1% β-mercaptoethanol, 1 mM phenylmethanesulfonylfluoride (PMSF), and 10 mM EDTA was treated for 2 minutes at 95°C. Each product was then diluted 2.5-fold with PBS containing 1% Nonidet P-40 (NP40) and mixed with peptide-*N*-glycosidase F (PNGase F, specific activity 25,000 units/mg, final concentration 1 or 0.1 μg/ml as indicated at the bottom of the figure). Mixtures were then incubated 20 h at 37°C (lower panels) or for various times (upper panel) as indicated. The enzymatic reaction was stopped by boiling in reducing SDS-PAGE sample buffer.

cases. Of note, upon boiling the 29–31 kD bands (recombinant sTNF-R1) as well as the 31–33 and 48 kD bands (urinary sTNF-R1) decreased, but the >60 kD bands increased. When TNF-α was omitted or when an irrelevant rabbit antiserum was used in the detecting steps, instead of the anti-TNF-α antiserum, no staining was observed, suggesting that the staining was specific (not shown). We conclude, therefore, that the >60 kD bands in these gels are probably aggregates formed upon boiling.

The capability of the 31–33 and 48 kD proteins to bind TNF-α in ligand blotting experiments suggests that both bands correspond to TNF-α binding proteins. This hypothesis is strengthened by the fact that the 31–33 and 48 kD bands were also observed by SDS-PAGE analysis of urinary sTNF-R1 purified by method A (first peak in Fig. 1), including an affinity chromatography step on TNF-α-agarose.

In conclusion, the results of SDS-PAGE and western and ligand blot experiments suggest that urine contains 31–33 and 48 kD products that are immunologically related to TNF-R1 and are functionally able to bind TNF-α.

Characterization of sTNF-R1 glycosylation

To characterize better the urinary and CHO sTNF-R1 structures, the N- and O-linked sugar content of both products were analyzed. To this aim, both products were first treated with peptide-*N*-glycosidase and analyzed by reducing SDS-PAGE and western blotting with rabbit anti-sTNF-R1 antiserum. SDS-PAGE of N-deglycosylated products revealed identical 21 kD bands in both recombinant sTNF-R1 and urinary sTNF-R1 (Fig. 4A). However, an additional band of 36 kD was observed in the urinary product. This band was recognized by rabbit anti-sTNF-R1 antiserum in a western blot (Fig. 4, right), confirming that is related to sTNF-R1 and was present even when 10-fold more peptide-*N*-glycosidase F was used, suggesting that N deglycosylation was complete.

The carbohydrate content of each product after N deglycosylation was analyzed using a commercially available glycan detection kit (Boheringer Mannheim). As shown in Fig. 5, sugars were detectable in the 36 kD product but not in the 12 kD product.

The results of N deglycosylation studies, altogether, indicate that (1) the 29–31 kD recombinant as well as the 31–33 kD urinary forms are N-glycosylated; (2) the differences between the 29–31 and 31–33 kD recombinant and natural products are mainly related to differences in the N-linked sugar content; and (3) the 48 kD sTNF-R1 isolated from urine contain N-linked sugars as well as other sugars attached to sites different from asparagine.

O deglycosylation studies were then undertaken. Because it is known that removal of sialic acid with neuraminidase may favor deglycosylation with *O*-glycanase, a mixture of these

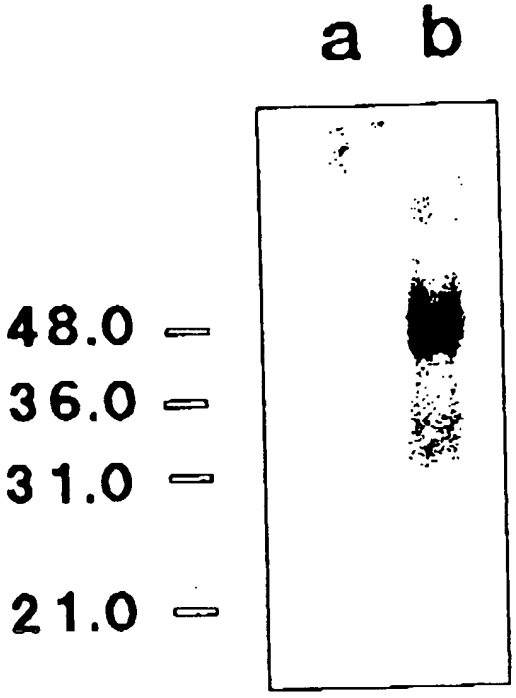

FIG. 5. Carbohydrate analysis of urinary sTNF-R1 treated and untreated with peptide-*N*-glycosidase F. Urinary sTNF-R1, treated (lane a) and untreated (lane b) with peptide-*N*-glycosidase F, was run by reducing SDS-PAGE and blotted on a nitrocellulose filter. Enzymatic treatment was carried out as described in Fig. 4. Detection of carbohydrates was carried out using the digoxigenin-hydrazide method.

FIG. 6. Molecular weight and carbohydrate analysis of urinary sTNF-R1 treated or untreated with neuraminidase and *O*-glycanase. (A) Reducing SDS-PAGE after silver staining; (B) staining with the digoxigenin-hydrazide method after blotting on nitrocellulose filters. Lane a, molecular weight markers (see Fig. 2); lane b, control with neuraminidase and *O*-glycanase alone; lane c, untreated urinary sTNF-R1; lane d, urinary sTNF-R1 treated with neuraminidase; lane e, urinary sTNF-R1 treated with neuraminidase and *O*-glycanase. Enzymatic treatments were carried out as follows: urinary sTNF-R1, 100 μg/ml, in 15 mM sodium phosphate buffer, pH 7.5, containing 0.1% wt/vol SDS, 0.5% β-mercaptoethanol, 1 mM PMSF, and 10 mM EDTA was treated for 5 minutes at 95°C. The product was then mixed with NP40 (1% vol/vol final concentration), vortexed for 1 minute, and mixed with neuraminidase (specific activity, 50 U/mg; final concentration, 1.5 U/ml) or distilled water. After 1 h incubation at 37°C, the products were mixed with *O*-glycanase (final concentration, 100 mU/ml) or distilled water and further incubated 20 h at 37°C. The enzymatic reaction was stopped by boiling in reducing SDS-PAGE sample buffer.

enzymes was used to verify the presence of O-linked sugars on urinary sTNF-R1.

Figure 6 shows the results of SDS-PAGE after silver staining (Fig. 6A) and specific staining of carbohydrates of blotted gel (Fig. 6B) before and after treatment with the enzyme mixture or with neuraminidase alone. As shown, the enzymatic treatment had no effects on the 31–33 kD form, confirming the absence of O-linked sugars in this form. At variance, a marked decrease of molecular mass of the 48 kD form, to about 31–36 kD (lane e), occurred after treatment with the enzyme mixture, strongly suggesting the presence of O-linked sugars in this form. A decrease in molecular mass and carbohydrate staining was also observed after treatment with neuraminidase alone (lane d), suggesting that sialic acid is present in the 48 kD form. Of note, although the 48 kD band is probably less than 5% of the total material loaded on gel, by silver staining, this band was more efficiently stained than the 31–33 kD band by the digoxigenin-hydrazide method (Fig. 6, lanes c). This probably reflect the different composition and high sugar content in the 48 kD glycoform.

Removal of N- and O-glycosidic carbohydrates from the 48 kD form was also attempted by adding peptide-*N*-glycosidase F to the neuraminidase and *O*-glycanase reaction mixture. However, this treatment produced a diffused band from 21 to 33 kD that was very difficult to interpret (not shown). It is possible that the reaction buffer was not optimal for all enzymes and that partial N- and O-deglycosylation leading to multiple forms was achieved.

TNF-α and TNF-β neutralizing activity of natural and recombinant sTNF-R1

To investigate whether differential glycosylation of soluble receptors could affect the relative binding of TNF-α and TNF-β, the cytotoxicity inhibitory properties of the urinary sTNF-R1 (31–33 and 48 kD), CHO sTNF-R1 (29–33 kD) and that of a nonglycosylated recombinant sTNF-R1 produced in *E. coli* (20 kD) were compared. The concentrations of all products were first measured by ELISA. Then, the cytotoxic activity of 500 pg/ml TNF-α and 200 pg/ml TNF-β solutions was measured in the presence of various amounts of each product. The measured bioactive TNF-α and TNF-β concentrations are shown in Fig. 7, and the sTNF-R1 concentrations sufficient to give 50% inhibition (IC_{50}) are reported in Table 1. As shown, urinary sTNF-R1, CHO sTNF-R1, and *E. coli* sTNF-R1 can antagonize TNF-α cytotoxicity with a similar potency, suggesting that glycosylation does not affect TNF-α inhibition. The picture changed with TNF-β. In this case, the urinary sTNF-R1

was sevenfold more potent than the *E. coli* sTNF-R1, suggesting that glycosylation could play a role in TNF-β recognition.

DISCUSSION

In this work we have compared the structure of natural and recombinant sTNF-R1. Natural sTNF-R1 was isolated from the urine of normal subjects, whereas the recombinant form was purified from CHO cells transfected with a cDNA fragment coding for the extracellular domain of TNF-R1, engineered to terminate with Ile-Glu-Asn as in urinary sTNF-R1.[18] The activity of both products was also compared with that of a commercial recombinant sTNF-R1 expressed in *E. coli*.

The urinary and CHO sTNF-R1 were found to be immunologically closely related and functionally active when analyzed by ELISA with various monoclonal antibodies and by a TNF-α cytotoxicity neutralization assay. However, when the purified products were analyzed by SDS-PAGE, western blotting, and ligand blotting with TNF-α, several differences in the apparent molecular weights were observed. In particular, analysis under reducing conditions of the urinary sTNF-R1 revealed a major diffused 31–33 kD band and a minor 48 kD band (the latter less than 5% of total), whereas only a 29–31 kD doublet was observed with recombinant sTNF-R1.

Several other bands, corresponding to multimers of the natural and recombinant forms, were also observed. Because we found that the number and the intensity of these bands were variable and dependent on the mode in which the samples were prepared, it is likely that these bands are related to aggregates. In particular, we found that the relative proportion of these bands increased in the absence of β-mercaptoethanol in the sample buffer and after boiling for a few minutes. Moreover, we observed an increase in these bands, even under reducing conditions, upon prolonged storage of the purified products, suggesting that these bands reflect irreversible denaturation and aggregation.

Previous studies on TNF-α binding proteins isolated from the urine of normal subjects and patients showed that the molecular

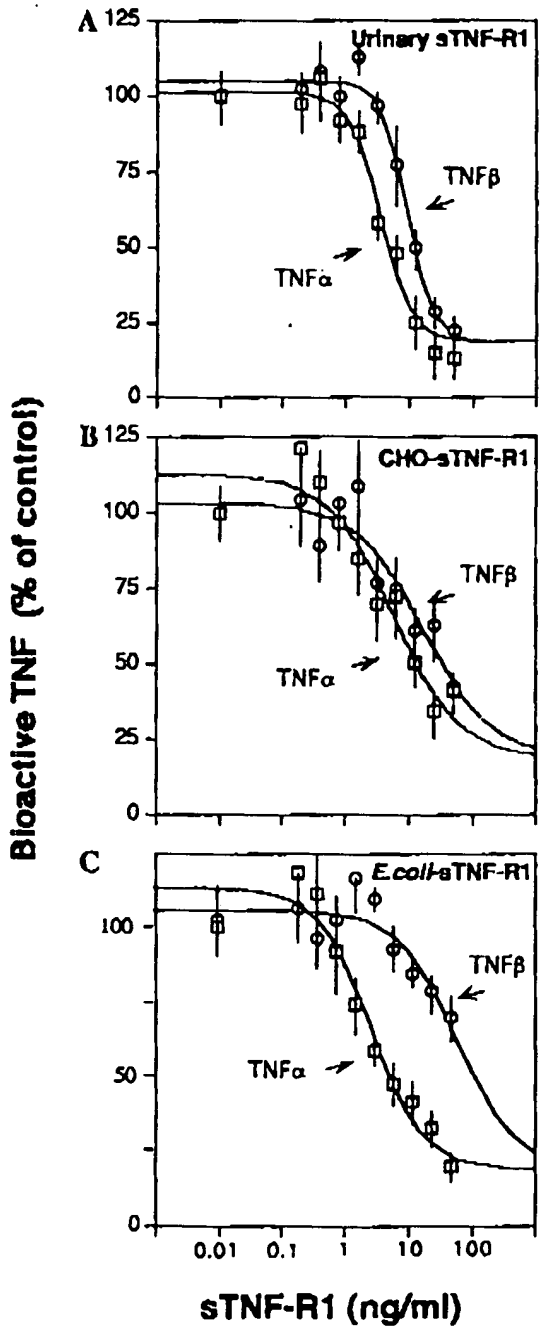

FIG. 7. Inhibition of TNF-α and TNF-β cytotoxicity (L-M cells) by urinary sTNF-R1 (A), recombinant sTNF-R1 expressed in CHO cells (B), and recombinant sTNF-R1 expressed in *E. coli* (C). Mouse L-M cells (ATCC CCL1.2) were cultured in minimum essential medium with Earle's salts (GIBCO), 5% FCS, and 2 mM glutamine (MEM-FCS) at 37°C, 5% CO_2. sTNF-R1 was incubated at various concentrations in the presence or absence of 500 pg/ml of TNF-α or 200 pg/ml of TNF-β, for 1 h at 37°C in MEM-FCS. Each mixture (50 μl) and actinomycin D (50 μl, 8 μg/ml) were then added in triplicate to L-M cells that had been seeded the day before at 30,000 cells/well in 96-well flat-bottomed plates in MEM-FCS. After overnight incubation at 37°C, live cells were stained with MTT (final concentration, 450 μg/ml) for 4 h. Supernatants were then aspirated, dimethylsulfoxide was added (200 μl/well), and the optical density of each well was read at 595 nm. Calibration curves for TNF-α and TNF-β cytotoxicity were prepared by testing, in parallel, six solutions of TNF-α or TNF-β alone at concentrations ranging from 0.031 to 1 ng/ml and by plotting absorbance values versus concentration. Bioactive TNF-α and TNF-β in the mixtures were calculated by interpolating the absorbance values obtained with each of the mixtures on the relevant calibration curve. Each point represents the mean ± SD (standard deviation) of three separate experiments. The curves were drawn using the ALLFIT program,[40] assuming that the minimum bioactive TNF is identical for all curves.